

Exhibit # 526

A PROGRESS REPORT ON THE EFFECTS OF METHANOL
ON THE MICROFLORA OF ARCTIC SOILS

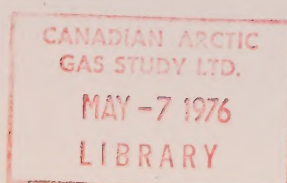
by

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for

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TABLE OF CONTENTS

	Page
Introduction -----	3
Study Area and Methanol Treatment -----	3
Aspects of Study -----	4
Methods -----	4
Results and Discussion -----	8
Summary -----	11
Future Aspects of Project -----	12
Notes -----	13
Literature Cited -----	13

Introduction

In the event of a pipeline failure during pipeline hydrostatic pressure testing, or a spill during storage or transportation, methanol could be released onto tundra soils. It is the aim of this study to assess the short- and long-term effects of methanol on soil microorganisms as well as to gain estimates on the rate of disappearance of methanol from the tundra soil. The research is also directed towards ascertaining the microbial and physical elements which affect the rate of disappearance of methanol.

To date the short-term effects of methanol on the bacterial component of the soil microflora have been completed and the results are included herein.

Study Area and Methanol Treatment

In 1973 an experimental study site was established near Inuvik, N.W.T. Younkin (1974) examined the effects of methanol on the vegetation and also provided a detailed site description. In February 1974 varying rates of 20% (by weight) methanol were applied to the plots. The rates ranged from 4 l/m^2 to 36 l/m^2 . In August 1975 a visit was made to this same experimental site. It was proposed that the first phase of this present study be confined to a laboratory examination of unamended soil which was to be sampled adjacent to the 1973 experimental site. Thus on this visit 120 soil cores, approximately 15 cm in length, were collected; subsequently 60 were frozen and 60 kept at 5°C . The unfrozen cores were used in the initial study so that the effect of freezing and thawing on soil populations would be eliminated and thus maintain the soil in as natural a

state as possible.

Aspect of the Study

To assess the short-term effects of methanol on the soil microflora the following areas were selected:

- i) Bacterial numbers
- ii) Amounts of fungal hyphae
- iii) Soil respiration
- iv) Assay of residual methanol
- v) Rate of volatilization and biodegradation of methanol
- vi) Organic matter content
- vii) Water content

Methods

The 60 cores which were unfrozen and kept at 5°C were used first and were carefully placed in plastic tubes (12 cm long x 2.5 cm diam). One end of the plastic tube was covered with nylon mesh and thus functioned as the bottom when the core was in an upright position. The following treatments were then applied:

1. To 20 of the cores 20% methanol was added (85.3 ml/core i.e., 36 litres/m²).
2. To 20 cores absolute methanol was added (85.3 ml/core).
3. To 20 cores distilled water was added (85.3 ml/core).

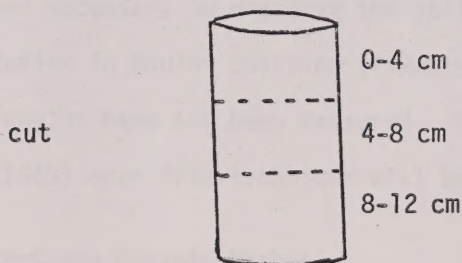
This treatment served as control.

The cores were placed in plastic bags and preconditioned at 15°C for 24 hours before applying the appropriate amount of methanol or water to them. It was hoped that conditioning the cores in this way would reduce water evaporation before the treatments were applied.

The methanol and water were applied very slowly and carefully with the aid of a graduated cylinder.

To assess the short-term effects after methanol application, several sampling times were chosen: i.e., 1 hour, 8 hours, 24 hours, 72 hours and 7 days after methanol application. At each sampling time 3 cores from each treatment were taken. Each core with the mesh on the bottom was placed upright on 3 paper towels (to absorb any excess methanol passing through the core). To allow for sufficient time for complete analysis (described previously) of each core, care was taken to stagger these analyses.

When required for analysis each soil core was removed from the plastic container and cut into 3 sections at 4 cm intervals.



Each 4 cm section was then cut into 4 subsections (each used for a specific analysis) according to the following scheme:

Bacteria	H ₂ O and Organic Content
Fungal Biomass	Residual Methanol

Bacterial Numbers

A 1 gram soil sample was taken from the appropriate subsection of each core and homogenized with 99 ml sterile dilute peptone water (0.25%) in an Osterizer for 3 minutes. Serial dilutions were made from this 1/100 diluted suspension and aliquots were plated onto dried peptone yeast extract agar plates (0.5% peptone, 0.1% yeast extract, 15% agar) with the aid of an alcohol flamed glass spreader. Three replicates of each of the dilutions 10^{-5} , 10^{-6} and 10^{-7} were plated from each soil sample. The plates were incubated at room temperature for 2 weeks and then counted.

Fungal Biomass

As it was impossible to make all required analyses immediately, it was found necessary to preserve the soil samples required for fungal biomass studies in Bouins solution (fixative). As yet the fungal mycelial lengths have not been measured. However, the Jones and Mollison (1948) agar film technique will be used.

Water Content and Organic Matter

A random amount of soil was placed into dried, preweighed crucibles. The weights were again recorded and then the crucibles containing the soil were placed in an oven at 105°C for 24 hours. At the end of this period the crucibles with dry soil were weighed. The dried soil and the weight of the crucible served as a base for the organic content analysis. The crucibles with the dried soil were placed in a muffle furnace at 375°C for 24 hours. At the end of this period the crucibles containing the non-organic residues were weighed.

Assay or Residual Methanol

1 gram samples of soil were each placed into 4 oz or 6 oz plastic screw cap containers containing 99 ml of water. The samples were immediately frozen until such time as analyses could be attempted. When analyses were begun, the bottles were thawed slowly at room temperature for a few hours. The liquid soil extract was then quickly poured into an osterizer and mixed for exactly 30 seconds. The mixed extract was then poured quickly back into the screw cap container. Four μ l of this solution were injected (using a 10 μ l syringe) into a Hewlett Packard (Model 5700A) gas-liquid chromatograph containing dual flame ionization detectors. A 6 ft 1/8 inch stainless steel Poropak Q column was used for the analysis. It was found by preliminary experimentation that the best results were obtained when the operational conditions were as follows: oven temperature 120°C, injection port 250°C, detector 250°C, carrier flow (nitrogen) 30 ml/min, hydrogen flow 30 ml/min, air flow 240 ml/min, injection volume 4 μ l, chart speed 1/2 in/min, range 10 and attenuation 32. All injections were done in triplicate.

The results were compared to a standard curve which was based on the following assay: 1 ml absolute methanol was diluted to 1 litre in a 1000 ml volumetric flask. Then, using the same syringe, 1 μ l, 2 μ l, 3 μ l, 4 μ l, 5 μ l, and 6 μ l were injected. The area under each methanol peak obtained could be converted to grams of methanol, knowing the specific gravity of absolute methanol diluted in this way is 1 g/ml.

Soil Respiration, Rate of Volatization and Biodegradation

This phase of the project is destined for analysis in the early part of 1976.

Results and Discussion

The means of the results for bacterial numbers, methanol analysis, water and organic matter content per gram dry weight are given in Table 1, along with their standard errors.

Organic Matter

The organic matter content remains extremely high throughout the first week indicating that little degradation has taken place (i.e., of the organic matter) during this time and that there is no likely relationship between organic matter content and bacterial numbers.

Water Content

It should be noted that with time the cores dried out in the cold room. As can be seen by viewing Table 1, there is a direct relationship between water content and bacterial numbers. This is extremely important and suggests that the results obtained for each time interval are relative only to their respective controls. This progressive soil drying is a feature of the experimental procedure which is being immediately corrected.

Bacterial Numbers

The results for bacterial numbers and presence of residual methanol have been compared at three depths (i.e., 0-4 cm, 4-8 cm, and 8-12 cm) and two concentrations (20% and 100% methanol) with time (see Figures 1-6).

1. 20% Methanol

The same general patterns for methanol versus general bacterial numbers can be seen in Figures 1 to 3. It would seem that

within the first day of application there is a marked decrease in bacterial numbers at all three depths. It seems though that this decrease is at its lowest point after 24 hours and that between 3 days and 1 week the bacterial numbers increase back to the level of the controls, suggesting that as the residual methanol volatilizes to a small enough level the bacterial numbers recover from the effects of the original application.

2. 100% Methanol

The general trend seems to be that after 72 hours, the methanol causes a decrease in numbers and that after 7 days this effect is still prominent. However, it should be noted that Figures 5 and 6 suggest that the effect of the methanol has been somewhat overcome after 7 days.

Interesting Sideline

A particularly interesting point emerged from the bacteria plated at 72 hours and 1 week following methanol treatment, i.e., the unusual predominance of yellow mucoid bacterium. By referring to Figures 7, 8, 10 and 11 it could be said that the 20% methanol treatment seems to have had little effect on the populations, as yellow and white colours of bacteria can be easily seen in apparently the same relative proportions, although a general decrease in numbers between controls and treated cores is easily seen in Figures 7 and 8.

The results in Figures 10, 11 and 12 are easier to interpret due to the dilution range used (i.e., 10^{-6} as compared to 10^{-5} at 72 h). Again the controls show a population comprised of mostly yellow bacterial colonies and a few genera of white bacteria. This relation-

ship still seems apparent when compared to the 20% treatment (i.e., compare Figure 10 and Figure 11). However, when Figure 10 is compared with Figure 12 one sees that a great deal of the white bacteria have been lost and almost a pure culture of yellow bacteria remains. This is the first evidence to point out with some certainty that 100% methanol treatment of soil has deleterious effects on some bacterial species but the major components of the population (i.e., yellow bacteria) seem to survive the treatment.

The presence of this yellow bacterial species in such high proportion demanded a closer study. In order to ascertain its identity, a yellow colony was randomly selected from each replicate and plated out into pure culture on peptone yeast extract agar plates. The gram reaction was strongly negative. The length was approximately $.79 \mu$. All cultures gave an acid reaction to glucose and a negative reaction to lactose when grown on Hugh and Leifson's media. All replicates were non motile and all yellow bacteria colonies observed had the physical characteristics of being round, shiny yellow, raised, and translucent to opaque. These results tentatively placed the bacteria as belonging to the genus *Flavobacterium* and probably to the species *lutescens*.

It was decided to further test this species in pure culture. When a growth curve had been established, a culture was taken in mid logarithmic growth and subjected to a concentration of methanol that would be equivalent to the concentrations of methanol actually existing in the soil cores. The estimates on soil water content imply that the concentration of 100% methanol was diluted to 25% and that 20%

methanol was diluted to approximately 6%. The correct amount of methanol was thus added to the actively growing culture. In each treatment a separate control was used and the equivalent amount of sterile dilute peptone water was added respectively. At the same time intervals as used for the soil cores, aliquots of the treated cultures along with their controls were plated out on peptone yeast extract agar plates. Also, Klett units were measured.

The results of this study are not yet complete but the Klett readings indicate that the culture with 6% methanol still grows, although its rate is significantly reduced. The culture treated with 25% methanol does not grow. What is interesting though is that the bacterial species is still viable, for when plated on peptone yeast extract agar plates, yellow colonies appear. It has not been determined if there is any variation in numbers with time (plates are still incubating).

What emerges from this test is a result which may be interpreted as optimistic. These pure cultures with their applied concentrations of methanol were kept in closed screw cap erlenmeyer flasks equipped with an extendable arm for measuring Klett units. The applied methanol had no place to evaporate so that the pure culture was probably subjected to more severe conditions than the soil cores used in the study, and yet there were viable colonies.

Summary

1. 20% methanol applied to cores seems to have an immediate effect causing a general decrease in bacterial numbers.
2. After 1 week it seems that the bacterial population has regained its status and overcome the effects of 20% methanol (Figures 1,

- 2 and 3). Also it seems that 20% methanol does not have a drastic effect in causing population shifts (Figures 7, 8, 10 and 11).
3. 100% methanol applied to cores seems to have a more long-lasting effect on general bacterial numbers (Figures 4, 5 and 6) and seems to cause some population shifts (compare Figures 10 and 12).
 4. The major component of the bacterial population (yellow) has been tentatively identified as *Flavobacterium lutescens*.
 5. In pure culture, with the appropriate concentration of methanol applied to *Flavobacterium lutescens*, it seems that 6% methanol is not greatly damaging but it does decrease the growth rate. However, 25% methanol neither increases the growth rate, nor kills the population.

Future Aspects of the Project

1. To analyze the fungal hyphae preserved in Bouins solution.
2. To design a system to assess the rate of volatilization of methanol.
3. To examine soil respiration.
4. To determine bacterial numbers, methanol, water, organic content and fungal biomass over a period of 4 weeks. I have attempted this experiment once but had problems with my soil cores drying out.
5. Next summer (Aug. 76) to examine field data which consists of analysis of soil exposed to methanol for 1 and 2 years.

Notes

I suspect that the methanol is being volatized from the bottom of the core as well as from the surface, the way the present system was set up (i.e., cores in upright position on top of 3 paper towels). It was set up this way originally so that any methanol that passed through the system would be removed, thus eliminating pools of methanol at the bottom of the core. If one examines the results in Table 1 and proceeds down the column (i.e., from 0-4 cm to 4-8 cm to 8-12 cm) one sees that after 72 hours and 1 week there is less methanol at 8-12 cm than at 4-8 cm. This does not seem natural for it would seem that the greater in depth in the soil one goes the less chance for volatization of methanol. The issue may possibly be resolved by allowing the bottom 4 cm to act as a reservoir for excess methanol and assume that the critical area of study is the top 8 cm.

The pure culture study does not strongly indicate that the methanol is being used as a substrate for growth so it would seem that certain bacteria can tolerate mass doses of methanol. (This point needs further proof and a future study may ratify this.)

Literature Cited

Younkin, W. 1974. Effects of winter methanol spills on Forest tundra vegetation. Report prepared by N.E.S.C.L. for C.A.G.S.L.

Table 1

Time Depth 0-4 cm	1 Hour			8 Hours			24 Hours			72 Hours			1 Week		
	Control	20%	100%	Control	20%	100%	Control	20%	100%	Control	20%	100%	Control	20%	100%
Bacteria x 10 ⁷ gram dry weight	82 ± 8	224 ± 47	114 ± 4	90 ± 20	227 ± 112	48 ± 13	71 ± 15	34 ± 4	76 ± 12	36 ± 1	18 ± 4	24 ± 5	33 ± 6	36 ± 13	15 ± 3
grams MeOH gram dry weight	0	.18 ± .02	1.12 ± .18	0	.34 ± .03	1.4 ± .4	0	.19 ± .02	.53 ± .12	0	.08 ± .01	.28 ± .02	0	.02 ± 0	.04 ± .01
grams H ₂ O gram dry weight	5.2 ± .48	3.68 ± .31	2.86 ± .19	5.74 ± .4	5.79 ± .6	3.4 ± .6	3.5 ± .3	3.49 ± .25	2.62 ± .2	2.36 ± .08	2.78 ± .24	2.11 ± .14	1.93 ± .28	2.16 ± .33	1.16 ± .01
grams organic matter gram dry weight	-	-	-	.89 ± .02	.95 ± .02	.94 ± .02	-	.91 ± .01	.94 ± .01	.91 ± .01	.94 ± .02	.94 ± .01	.91 ± .02	.88 ± .01	.92 ± .01
4-8 cm															
Bacteria x 10 ⁷ gram dry weight	43 ± 2	45 ± 9	50 ± 7	57 ± 6.4	61 ± 8	33 ± 3	50 ± 7	27 ± 2	41 ± 2	25 ± 3	13 ± 2	12 ± 2	19 ± 1	29 ± 2	.13 ± 5
grams MeOH gram dry weight	0	.21 ± .02	1.04 ± .16	0	.26 ± .03	1.3 ± .11	0	.25 ± .15	.68 ± .09	0	.15 ± .01	.55 ± .04	0	.05 ± .01	.13 ± .01
grams H ₂ O gram dry weight	5.55 ± .52	4.09 ± .11	3.67 ± .27	5.5 ± .4	5.01 ± .5	3.8 ± .5	3.7 ± .1	4.02 ± .1	2.5 ± .02	2.85 ± .5	2.87 ± .11	2.08 ± .16	2.57 ± .18	2.41 ± .07	1.59 ± .01
grams organic matter gram dry weight	-	-	-	.93 ± .01	.95 ± .01	.93 ± .01	-	.88 ± .06	.94 ± 0	.93 ± .02	.94 ± .01	.93 ± .01	.94 ± .01	.91 ± .01	.92 ± .01
8-12 cm															
Bacteria x 10 ⁷ gram dry weight	50 ± 1	38 ± 1.3	55 ± 3	52 ± 4	54 ± 12	34 ± 5	47 ± 4	32 ± 8	31 ± 9	22 ± 5	8 ± 1	11 ± 2	15 ± 1	27 ± 4	12 ± 2
grams MeOH gram dry weight	0	.11 ± .03	1.12 ± .07	0	.26 ± .02	1.03 ± .1	0	.28 ± .05	.54 ± .1	0	.07 ± 0	.33 ± .08	0	.03 ± .01	.09 ± .04
grams H ₂ O gram dry weight	4.93 ± .27	4.35 ± .41	6.01 ± 1.2	5.9 ± 0	5.85 ± .5	4.3 ± .14	3.8 ± .2	6.38 ± 1.1	3.1 ± .4	2.98 ± .5	2.49 ± .18	2.02 ± .09	2.07 ± .02	1.99 ± .09	1.51 ± .01
grams organic matter gram dry weight	-	-	-	.94 ± .02	.97 ± .01	.94 ± .01	-	.90 ± .06	.94 ± .01	.93 ± .01	.95 ± .01	.93 ± .01	.93 ± .01	.89 ± .01	.92 ± .01

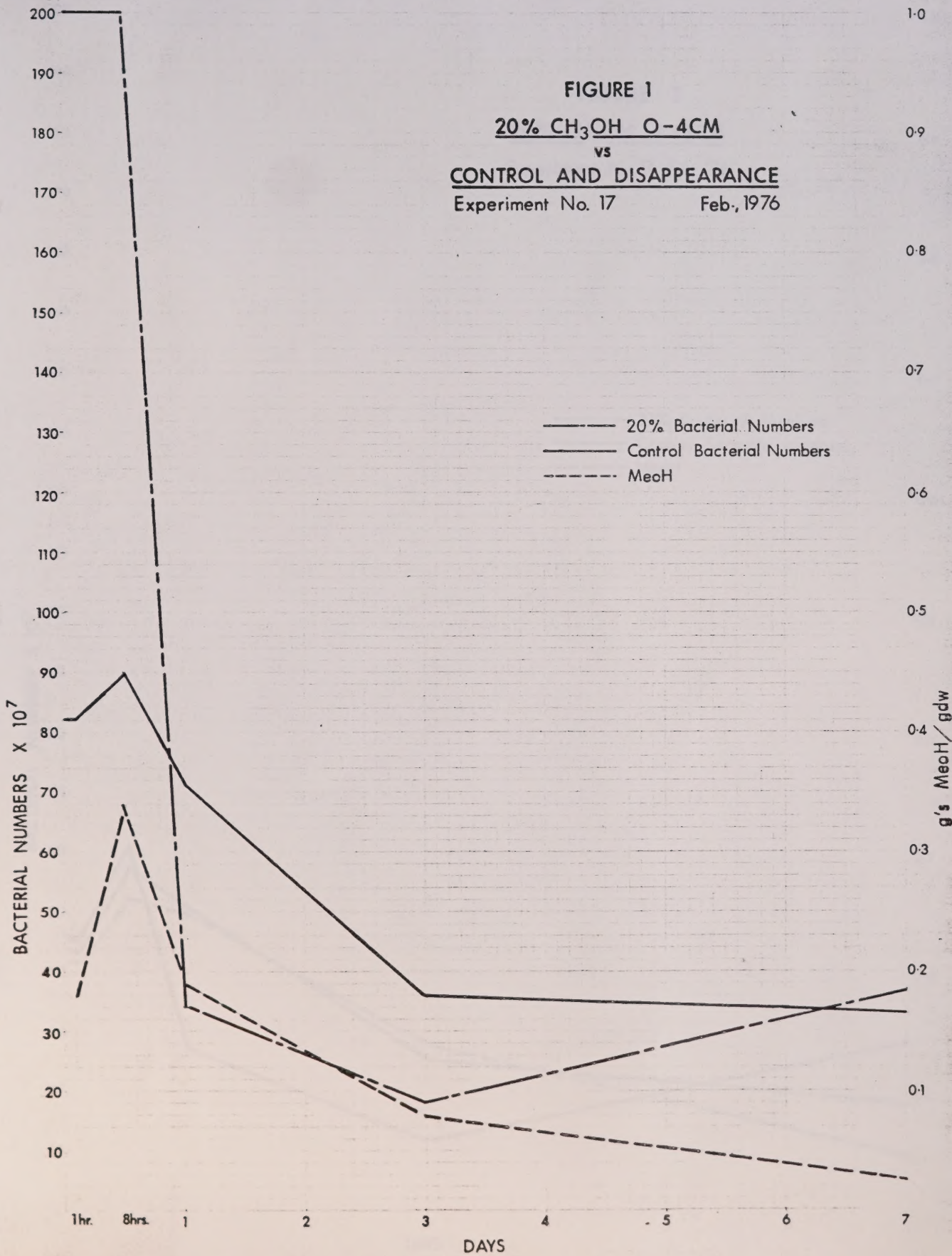
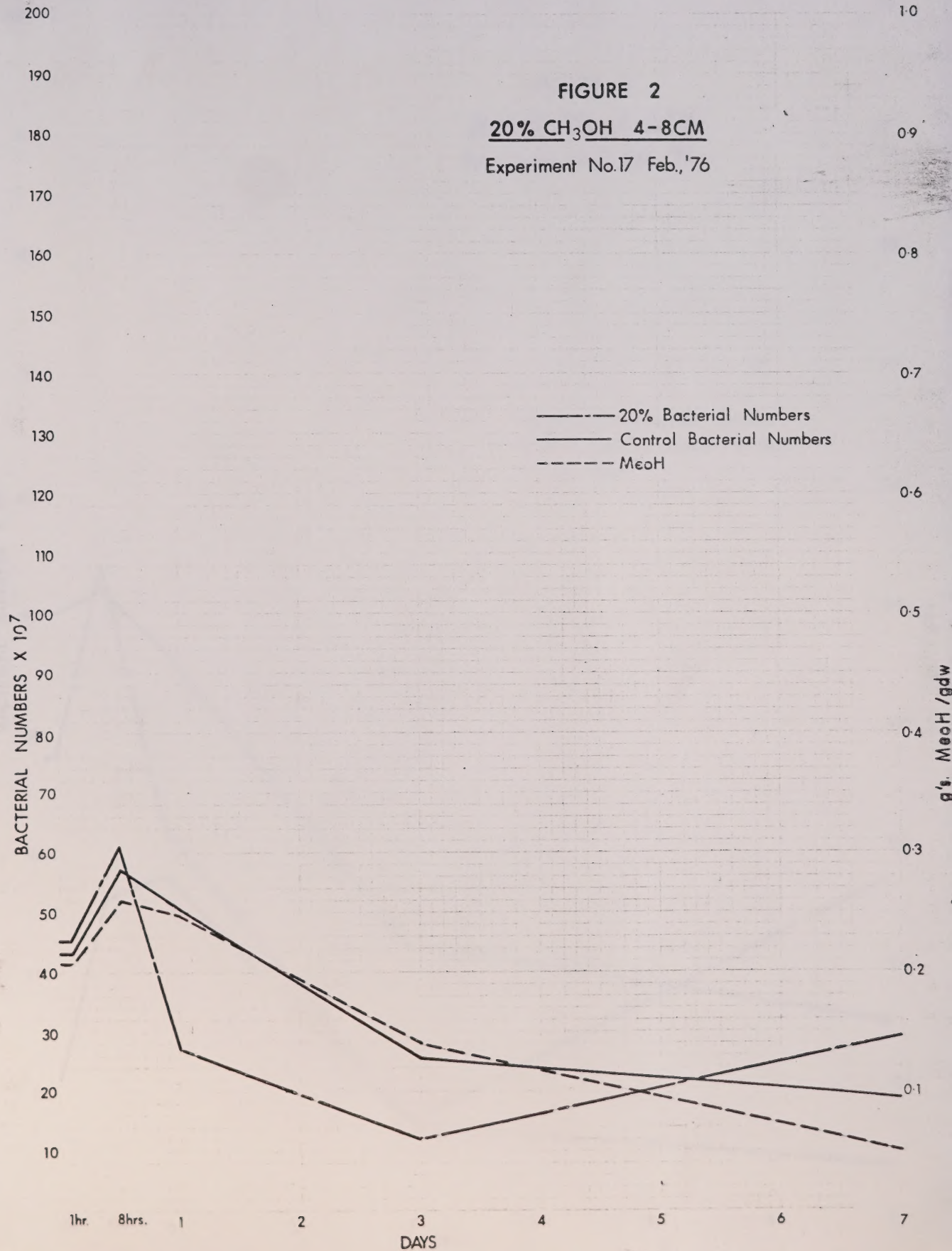


FIGURE 2

20% CH₃OH 4-8CM

Experiment No.17 Feb., '76



100

1.0

FIGURE 3

20% CH₃OH 8-12 CM

Experiment No.17 Feb., '76

90

0.9

80

0.8

70

0.7

60

0.6

50

0.5

40

0.4

30

0.3

20

0.2

10

0.1

BACTERIAL NUMBERS X 10⁷

g's MeOH/gdw

— 20% Bacterial Numbers
— Control Bacterial Numbers
- - - MeOH

1hr.

8hrs.

1

2

3

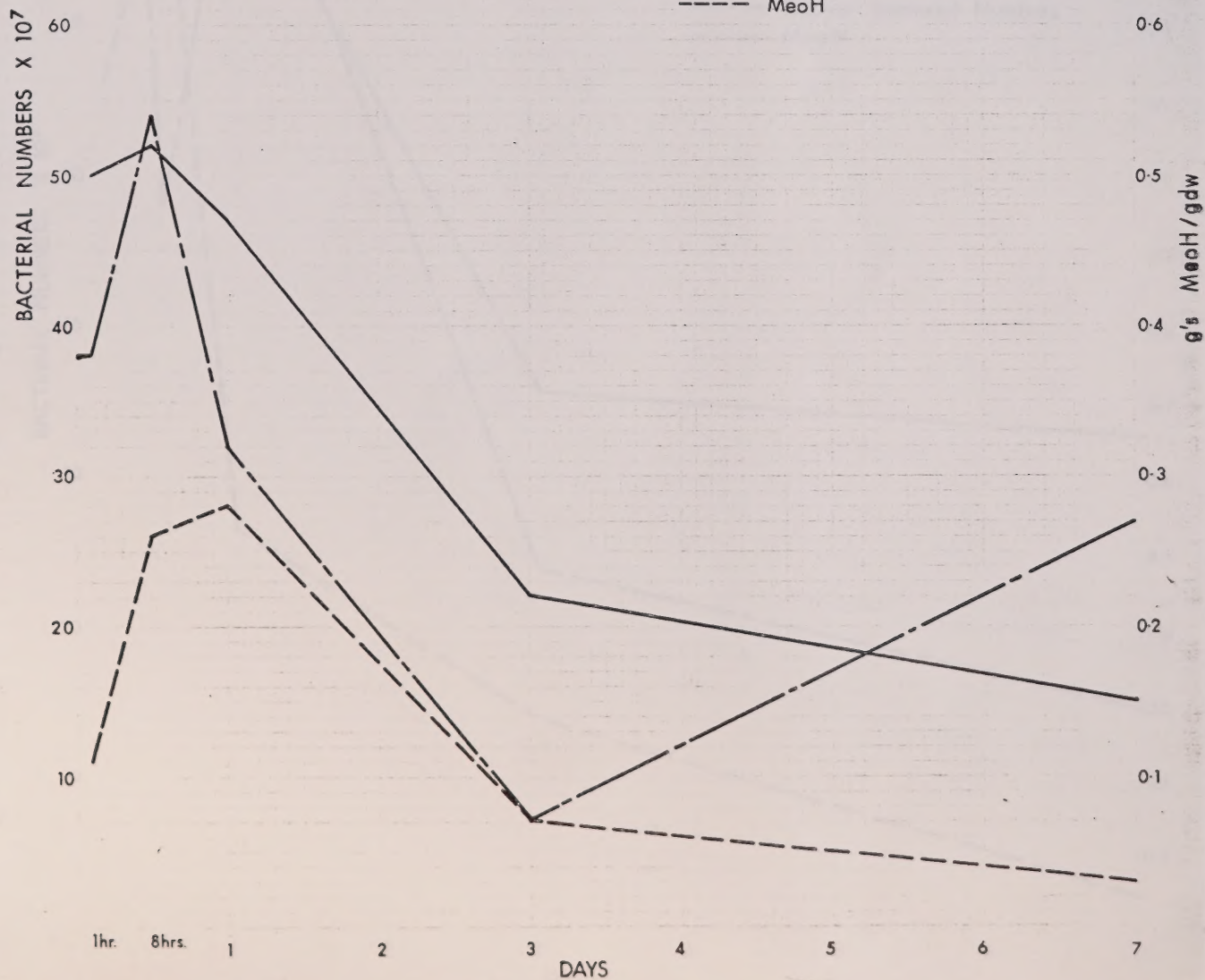
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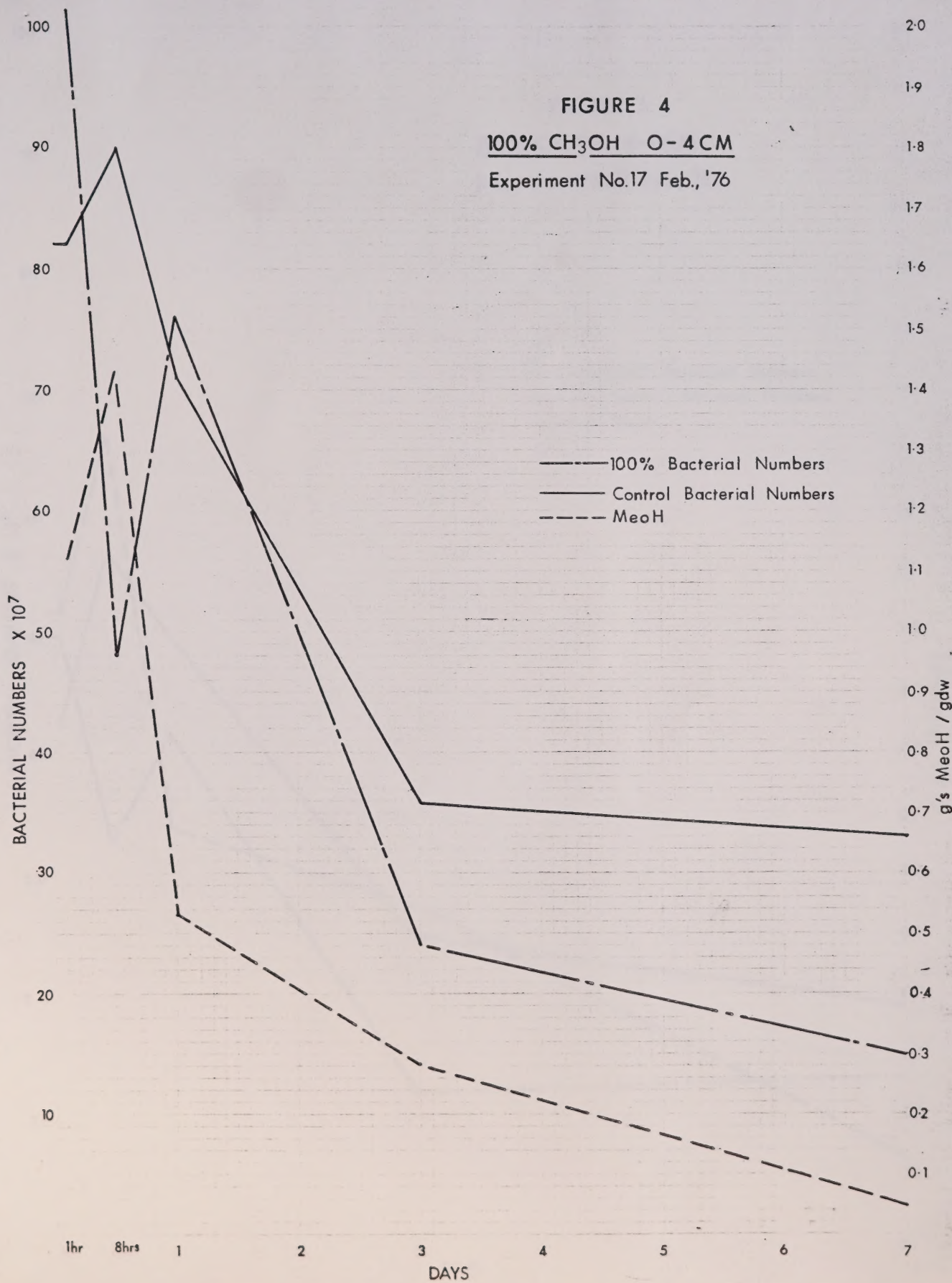
4

5

6

7





100

2.0

FIGURE 5

100% CH₃OH 4-8 CM

Experiment No.17 Feb., '76

1.9

1.8

1.7

1.6

1.5

1.4

1.3

1.2

1.1

1.0

0.9

0.8

0.7

0.6

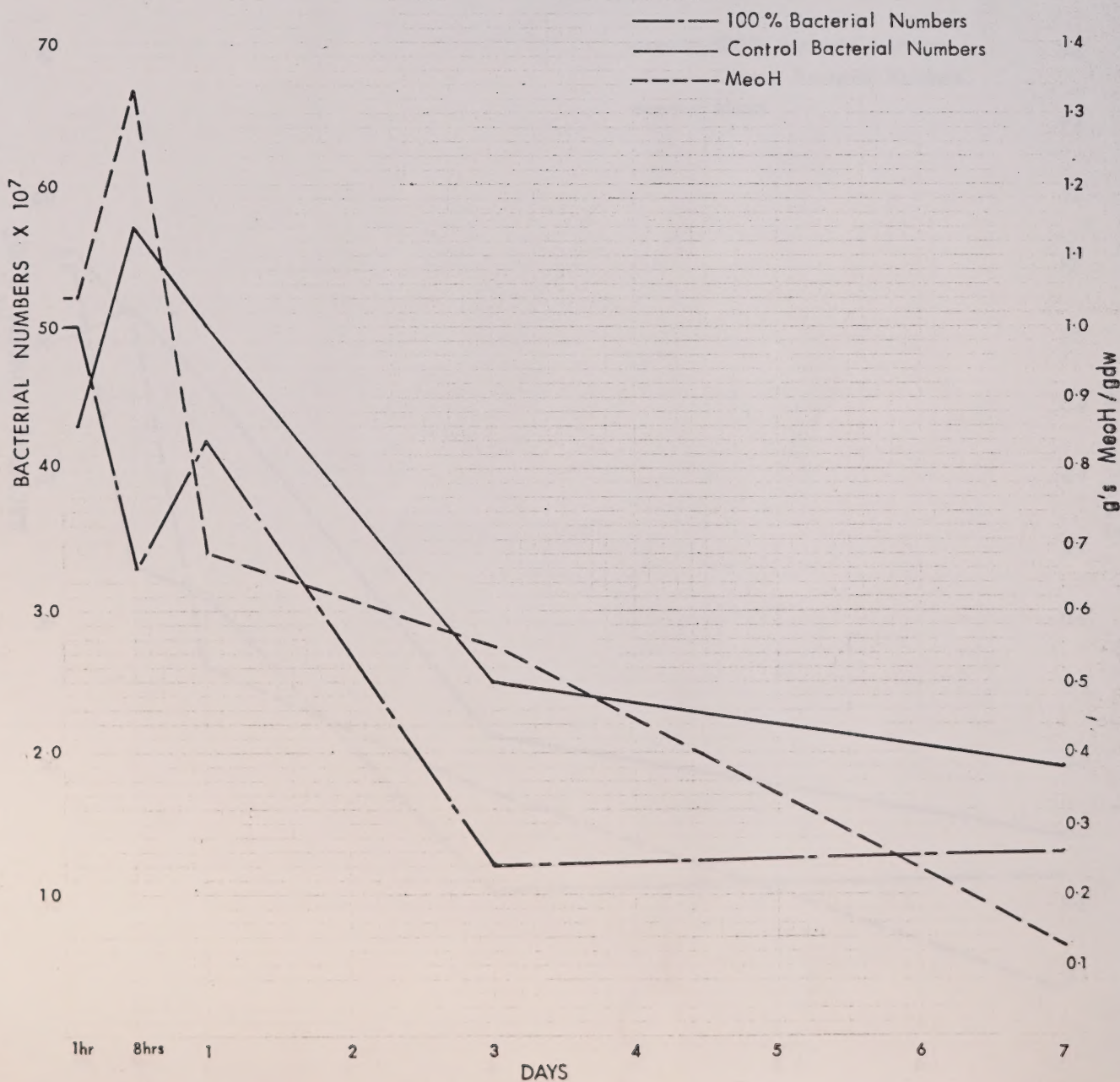
0.5

0.4

0.3

0.2

0.1



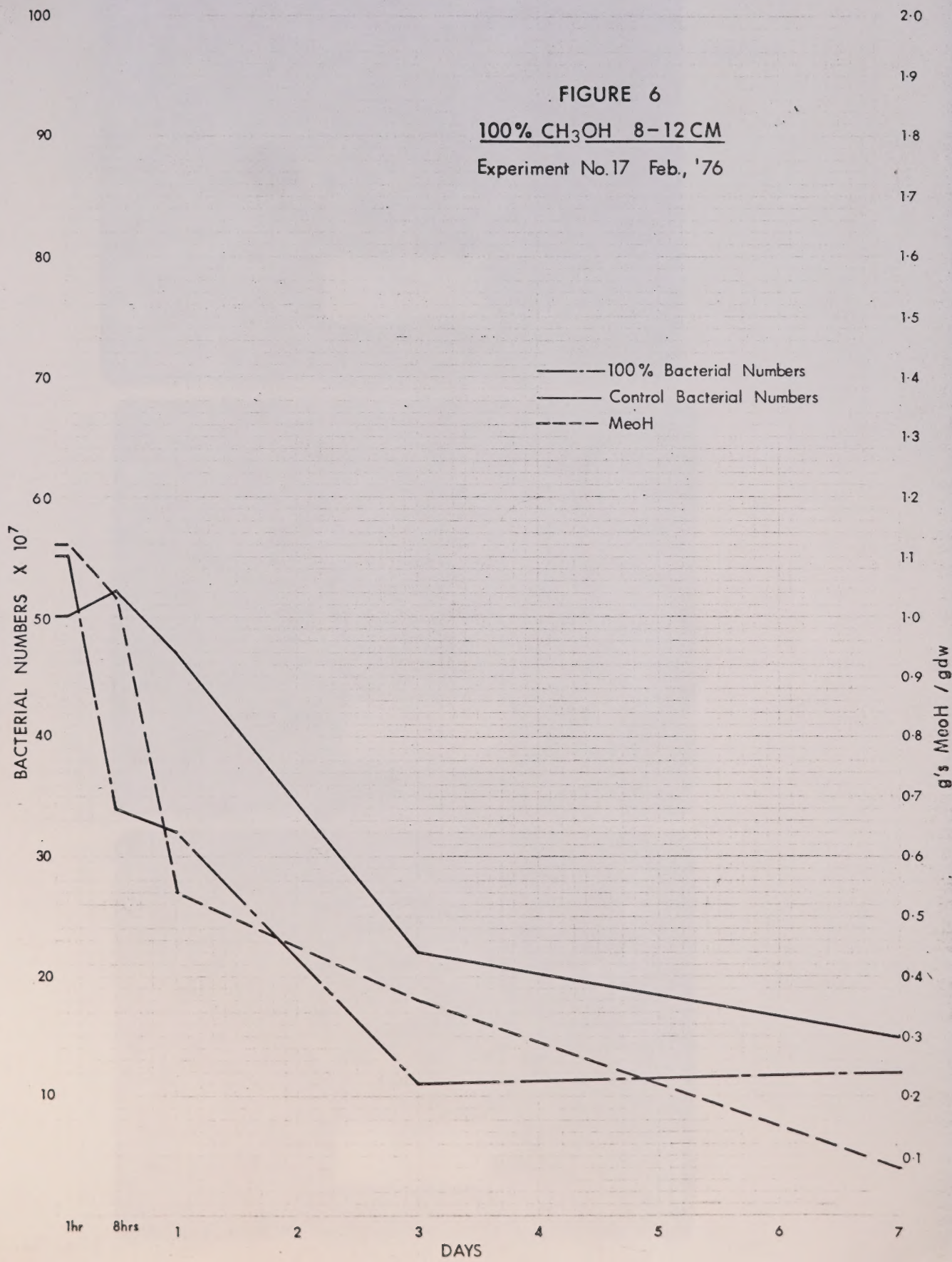


Figure 7



2 Control
10⁻⁵ dil'n
0-4 cm
72 hrs



2 Control
10⁻⁵ dil'n
4-8 cm
72 hrs

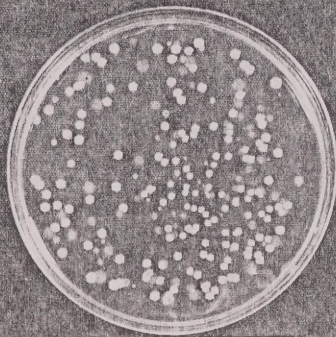


3 Control
10⁻⁵ dil'n
8-12 cm
72 hrs

Figure 8



3. 20% MeOH
10⁻⁵ d/l'n
0-4 cm
72 hrs



3. 20% MeOH
10⁻⁵ d/l'n
4-8 cm
72 hrs



I. 20% MeOH
10⁻⁵ d/l'n
8-12 cm
72 hrs

Figure 9



2. Absolute MeOH
10⁻⁵ dil'n
0.4 cm
72 hrs



1. Absolute MeOH
10⁻⁵ dil'n
4-8 cm
72 hrs



2. Absolute MeOH
10⁻⁵ dil'n
8-12 cm
72 hrs

Figure 10



5 Control
10⁻⁶ dil'n
0-4 cm
1 wk



3 Control
10⁻⁶ dil'n
4-8 cm
1 wk



2 Control
10⁻⁶ dil'n
8-12 cm
1 wk

Figure 11



2 20% MeOH
10⁻⁶ diln
0-4 cm
1 wk

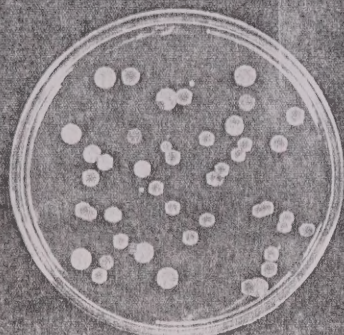


3 20% MeOH
10⁻⁶ diln
4-8 cm
1 wk



2 20% MeOH
10⁻⁶ diln
8-12 cm
1 wk

Figure 12



2. Absolute MeOH
 10^{-6} d.f.n.
0-4 cm
1 wk



3. Absolute MeOH
 10^{-6} d.f.n.
4-8 cm
1 week



2. Absolute MeOH
 10^{-6} d.f.n.
8-12 cm
1 wk

